

## PREVALENCE OF ANTIBODIES TO *NEOSPORA CANINUM*, *SARCOCYSTIS NEURONA*, AND *TOXOPLASMA GONDII* IN WILD HORSES FROM CENTRAL WYOMING

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**ABSTRACT:** *Sarcocystis neurona*, *Neospora caninum*, *N. hughesi*, and *Toxoplasma gondii* are 4 related coccidians considered to be associated with encephalomyelitis in horses. The source of infection for *N. hughesi* is unknown, whereas opossums, dogs, and cats are the definitive hosts for *S. neurona*, *N. caninum*, and *T. gondii*, respectively. Seroprevalence of these coccidians in 276 wild horses from central Wyoming outside the known range of the opossum (*Didelphis virginiana*) was determined. Antibodies to *T. gondii* were found only in 1 of 276 horses tested with the modified agglutination test using 1:25, 1:50, and 1:500 dilutions. Antibodies to *N. caninum* were found in 86 (31.1%) of the 276 horses tested with the *Neospora* agglutination test—the titers were 1:25 in 38 horses, 1:50 in 15, 1:100 in 9, 1:200 in 8, 1:400 in 4, 1:800 in 2, 1:1,600 in 2, 1:3,200 in 2, and 1:12,800 in 1. Antibodies to *S. neurona* were assessed with the serum immunoblot; of 276 horses tested, 18 had antibodies considered specific for *S. neurona*. Antibodies to *S. neurona* also were assessed with the *S. neurona* direct agglutination test (SAT). Thirty-nine of 265 horses tested had SAT antibodies—in titers of 1:50 in 26 horses and 1:100 in 13. The presence of *S. neurona* antibodies in horses in central Wyoming suggests that either there is cross-reactivity between *S. neurona* and some other infection or a definitive host other than opossum is the source of infection. In a retrospective study, *S. neurona* antibodies were not found by immunoblot in the sera of 243 horses from western Canada outside the range of *D. virginiana*.

*Sarcocystis neurona*, *Neospora caninum*, *N. hughesi*, and *Toxoplasma gondii* are related coccidians that are reported to cause encephalitis in horses. Of these 4 parasites, *S. neurona* is the most frequent cause of equine protozoal myeloencephalitis (EPM) in horses in the United States (Dubey et al., 1991; Dubey, Lindsay et al., 2001). The opossum (*Didelphis virginiana*) is the only known definitive host for *S. neurona* in the United States, and the distribution of EPM coincides with the geographic range of *D. virginiana*. Horses become infected by ingesting *S. neurona* oocysts or sporocysts excreted in feces of the opossum (Fenger et al., 1997; Dubey and Lindsay, 1998). More than 30% of horses tested in the United States were found to have antibodies to *S. neurona* by using immunoblot tests (IBTs) (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Tillotson et al., 1999; Rossano et al., 2001; Daft et al., 2002).

Antibodies to *N. caninum* or *N. hughesi* or both were found in 23.3% of 296 horses from 2 meat packing plants in Nebraska and Texas (Dubey, Romand et al., 1999), in 11.5% of 536 horses in Alabama (Cheadle et al., 1999), in 11% of 208 horses from Missouri (Vardeleon et al., 2001), and in 23% of 99 horses

from France (Pitel et al., 2001). Clinical neosporosis has been reported in a few horses only (Dubey and Porterfield, 1990; Daft et al., 1996; Gray et al., 1996; Lindsay et al., 1996; Marsh et al., 1996; Hamir et al., 1998; Cheadle et al., 1999; Pronost et al., 1999). The domestic dog (*Canis familiaris*) is the only known definitive host for *N. caninum* (McAllister et al., 1998); the definitive host for *N. hughesi* is unknown. Although *Neospora* spp. can be transmitted congenitally in horses (Dubey and Porterfield, 1990; Pronost et al., 1999), congenital equine neosporosis is considered a rare occurrence.

Before the discovery of *S. neurona* and *N. caninum*, clinical cases of EPM in horses were misdiagnosed as toxoplasmosis (reviewed in Dubey and Beattie, 1988; Dubey, Kerber et al., 1999). Compared with *S. neurona* and *N. caninum*, the seroprevalence of *T. gondii* in horses is low, and there is no proven case of clinical toxoplasmosis in horses. Antibodies to *T. gondii* were found in 6.9% of the 1,000 horses from the United States (Dubey, Thulliez et al., 1999). Felids are the definitive hosts for *T. gondii* (Dubey and Beattie, 1988).

The objective of the present study was to determine the seroprevalence of *S. neurona*, *N. caninum*, and *T. gondii* in wild horses from central Wyoming, outside the known geographic range of the opossum.

### MATERIALS AND METHODS

#### Wild horses from Wyoming

Serum samples were obtained from 276 wild horses stationed at Honor Farms, near Riverton in central Wyoming (latitude 43°01'30"N, longitude 108°22'30" N). These horses were caught in the wild and kept on this farm before private adoption. The average stay of horses on this farm was no more than 4 mo. While on the farm, horses were fed alfalfa hay and occasionally grain grown on the farm. Opossums have not been observed in this area or in the areas from which the horses were captured. Data regarding approximate age and sex were available for most horses. One hundred and thirty-seven females and 139 males were included.

#### Serologic testing

Blood samples were obtained from a jugular vein, and serum samples were sent to the Animal Parasitic Diseases Laboratory (APDL), United

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TABLE I. Antibodies to *Neospora caninum* in Wyoming horses of different ages.

Age (yr)	No. of horses	No. of horses with NAT titers of					Total positive
		25	50	100	200	≥400	
<1	29	4		2	2		8
1	21	3				2	5
2	49	6	4	2		3	15
3	37	7	1	2			10
4	28	6	3			1	10
5	9	2	1			1	4
6	13	2		1	2		5
7	6						0
8	2	1					1
9	7			2	1		3
10	6		1		1	1	3
11	2						0
12	12	2	2			2	6
13	8	1				2	3
14	13	1				2	3
15	7				1	1	2
16	3						0
18	2						0
20	6				1		1
22	3		1			1	2
25	1						0
Unknown	12	3	2				5
Total	276	38	15	9	8	16	86

States Department of Agriculture, Beltsville, Maryland, for serologic testing.

Antibodies to *T. gondii* and *N. caninum* were determined at APDL using direct agglutination tests. For *T. gondii*, sera were diluted 1:25, 1:50, and 1:500 and tested with the modified *T. gondii* agglutination test (MAT), according to a previously published protocol (Dubey and Desmonts, 1987). For *N. caninum*, sera were initially screened at 1:25, 1:50, and 1:500 dilutions using *Neospora* agglutination test (NAT), as described by Romand et al. (1998). Seropositive samples were titrated 2-fold, starting at 1:100 dilution.

Antibodies to *S. neurona* were determined by immunoblots as described (Granstrom et al., 1993; Dubey, Lindsay et al., 2001) and by *S. neurona* direct agglutination test (SAT) as described by Lindsay and Dubey (2001). The SAT was performed at the Center for Molecular Medicine and Infectious Diseases, Virginia Tech, Blacksburg, Virginia. The immunoblots were performed at Equine Biodiagnostics Inc. (EBI), Lexington, Kentucky. Antibodies (immunoglobulin G, IgG) to *S. neurona* were essentially detected by immunoblot as described previously (Granstrom et al., 1993). Essentially, lysates of merozoites were subjected to electrophoresis through denaturing gradient polyacrylamide gels (sodium dodecyl sulfate, 10–20% polyacrylamide gel electrophoresis). After electrophoresis, separated proteins were electrically transferred to nitrocellulose paper by standard methods. Equine sera were incubated with the blot individually, and anti-*S. neurona* antibodies were detected using biotinylated anti-equine IgG followed by streptavidin–peroxidase conjugate and aminoethyl carbazole peroxide chromagen substrate.

#### Domestic horses from western Canada

During the course of studies on pathogenesis of *S. neurona* infection in horses (Saville et al., 2001; Sofaly et al., 2002), 243 horses from the Saskatchewan and Manitoba area, Canada, were screened for *S. neurona* antibodies to obtain negative horses for the experiments. Horses were obtained from these areas because they are outside the range of *D.*

TABLE II. Data on 18 horses with confirmed antibodies to *Sarcocystis neurona* detected by immunoblot.

Horse no.	Age	Concurrent antibody titers by		
		NAT	SAT	MAT
61	1	<25	<50	<25
36	2	<25	<50	<25
22	2	<25	100	<25
94	2	<25	<50	<25
11	4	<25	<50	<25
22	4	25	100	<25
46	4	50	<50	<25
21	5	25	<50	<25
11	6	<25	50	<25
5	10	<25	<50	<25
104	10	50	50	<25
54	13	<25	50	<25
32	14	<25	<50	<25
59	15	800	<50	<25
37	18	<25	<50	<25
64	20	<25	50	<25
17	Unknown	25	100	<25
10	Unknown	<25	<50	<25

\*NAT = *Neospora* agglutination test, SAT = *Sarcocystis neurona* agglutination test, MAT = *Toxoplasma gondii* modified agglutination test.

virgiana. Results of immunoblots performed on these horses at EBI are included in the present article.

## RESULTS

### Wyoming horses

Antibodies to *N. caninum* were found in 86 (31.1%) of the 276 horses of all ages (Table I). Prevalences in females were similar to those in males. High antibody titers (≥1:400) were found in 18.6% of 86 seropositive horses; the highest titer was 1:12,800. Antibodies to *T. gondii* (MAT) were found in 1 horse, and the titer was only 1:25.

Antibodies to *S. neurona* were found by immunoblots in 18 of the 276 horses (Table II). By using the SAT, antibodies were found in 39 horses—in titers of 1:50 in 26 horses and 1:100 in 13. None of the 18 horses positive by immunoblot was positive by MAT; 6 were positive by NAT, and 7 were positive by SAT. Additionally, 15 horses exhibited equivocal (reported as weak) reactivity by immunoblot analysis. An illustration of the diversity of the immunoblot patterns is shown in a composite immunoblot (Fig. 1).

### Canadian horses

Among the 243 horses from western Canada, immunoblot analyses were negative for 219 horses (90.1%), weakly positive for 20 (8.2%), and confirmed positive for 4. It was later found that the 4 horses confirmed seropositive were raised in the United States.

## DISCUSSION

Clinical *N. caninum* and *N. hughesi* infections have been reported in horses in the United States and France. The distinction between these 2 parasites is based essentially on molecular

characteristics. The presence of shared antigens was demonstrated by the cross-reactivity in the NAT as well as in the indirect fluorescent antibody test (Marsh et al., 1996, 1998; Dubey, Liddell et al., 2001). Therefore, it is not possible to say whether the antibodies in 31% of wild horses from Wyoming were due to 1 or both parasites. These data indicate that the definitive host involved in the life cycle of equine *Neospora* spp. infections is present in central Wyoming. The 31% *Neospora* spp. seropositivity in horses of the present study is the highest reported to date. Previously, 23% of horses from the United States (Dubey, Thulliez et al., 1999) and France (Pitel et al., 2001) were reported to have *N. caninum* antibodies in the NAT. Whether the horses acquired *Neospora* spp. infections at the farm or in the wild is not known.

The NAT titer that should be considered specific for *Neospora* spp. infections has not been determined. Therefore, all titers are presented in the present study. The almost total absence of detectable *T. gondii* antibodies in horses with *Neospora* antibodies demonstrates the specificity of the NAT and MAT, confirming earlier findings in experimentally infected animals (Romand et al., 1998). Gupta et al. (2002) reported an indirect fluorescent antibody titer of 1:100 in a horse naturally infected with *N. hughesi*; they considered this finding to be the result of the cross-reactivity between these 2 organisms, but there is a possibility that the horse may have been exposed to both parasites.

The antemortem diagnosis of EPM remains a problem. The IBT was developed to detect the presence of *S. neurona*-specific antibodies in serum and cerebrospinal fluid (CSF). Although this test is not perfect, it is the best test available at present for the diagnosis of *S. neurona*. The IBT is highly specific if one considers weak positives as negatives, as supported by data obtained from Daft et al. (2002) and the present study with the horses from western Canada. Immunoblots from horses exposed to *S. falcatula* infections (Cutler et al., 1999) support the data on specificity of the IBT.

The lack of any apparent correlation between SAT and immunoblot in horses in the present study may be due to these tests measuring different antibodies. The SAT has not been evaluated for the diagnosis of EPM in experimentally infected horses or for use in clinical specimens.

Finding antibodies to *S. neurona* by immunoblot analysis in at least 18 horses native to Wyoming is unexpected and unexplained. Opossums are not known to occur in central Wyoming, and there has not been any confirmed case of EPM from horses native to Wyoming. Cross-reactivity with *N. caninum* was unlikely because 9 horses positive by *S. neurona* immunoblot had no detectable *N. caninum* antibody. Pitel et al. (2002) also reported *S. neurona* antibodies in horses native to France, although opossums do not occur in Europe.

As stated earlier, the IBT was developed by Granstrom et al. (1993) to detect *S. neurona*-specific antibodies in the serum and CSF of horses from the Western Hemisphere. This IBT is not known to cross-react with any related protozoan in limited investigations. Because it has not been possible to reliably induce verifiable EPM in horses, the only gold standard to evaluate the test is the verification of the parasite by postmortem immunohistochemical examination of the horses. Because it is not possible to rule out the absence of *S. neurona* by histologic examination of only a small amount

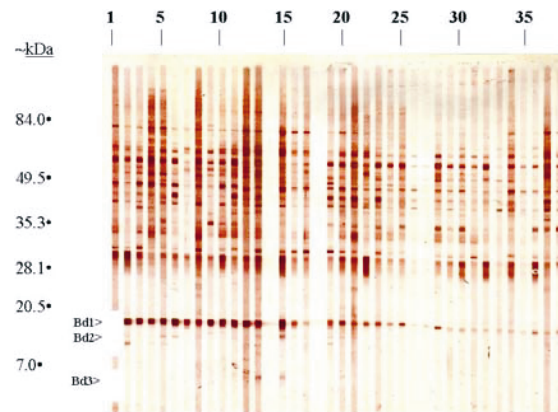


FIGURE 1. An immunoblot containing sera from a representative selection of the 276 study horses is shown. They were chosen on the basis of their immunoblot banding patterns and reactivities in the SAT assay. The *Sarcocystis neurona*-specific bands are designated by the numbered arrows to the left of the first lane, and their approximate molecular weights are based on the migration of standards of known molecular weight (far left). Band 1 = 14.5 kDa, band 2 = 12 kDa, and band 3 = <7 kDa. Lanes 15, 16, and 17 contain positive, diluted positive (for assessing lower detection limit), and negative sera, respectively. Lanes 14 and 18 are blank. Lanes 1–13 contain sera that tested positive by IBT. The sera in lanes 1, 8, and 11 also had SAT titers of 1:50, 1:100, and 1:100, respectively. Lanes 19–23 contain sera with equivocal IBT reactivity. Of these, the serum in lane 20 also had a SAT titer of 1:50. Lanes 24–38 contain sera that tested negative by IBT. The sera in lanes 24, 25, 27, 28, 29, 30, 31, and 37 had SAT titers of 1:100, 1:50, 1:100, 1:100, 1:100, 1:50, and 1:100, respectively. Of the 33 sera shown in this immunoblot, 15 also had titers in the *Neospora* agglutination test, ranging from 1:25 (lanes 8, 10, 12, 23, 31, 36, and 37) to 1:800 (lanes 5 and 30).

of central nervous system tissue (in grams), only a positive identification is meaningful. Recently, Daft et al. (2002) critically evaluated the performance of IBT in horses with neurologic diseases. They found that IBT was strongly positive in all 12 horses with confirmed *S. neurona* infection. More importantly, 8 horses negative for *S. neurona*-associated EPM were infected by other unidentified apicomplexans. Of these, 5 tested negative by IBT, but sera from 3 had weak reactivity in the IBT (Daft et al., 2002). It is difficult to interpret the significance of an equivocal result by testing a given horse at a single time point. For clinical specimens, it is recommended that a subsequent specimen (4–8 wk later) be tested and compared with the original result. Whereas some horses may progress to increased immunoreactivity, others may remain weak, probably representing a much earlier exposure to the parasite or, possibly, a very low level of nonspecific reactivity. Marsh et al. (1998) reported *S. neurona* immunoreactivity in a horse diagnosed with encephalitis due to *N. hughesi*. In light of the findings of the studies by Daft et al. (2002) and the present study and from the findings on other naturally infected horses (Dubey, Kerber, and Granstrom, 1999; Dubey, Venturini et al., 1999) and experimentally infected rabbits, there is no evidence that *S. neurona*-specific antibodies detected by IBT react with *Neospora* spp. antigens. It seems most likely that the horse was exposed to both parasites. The presence of *S. neurona*-specific antibodies in horses from central Wyoming is puzz-



zling and suggests that another definitive host may be involved or that the parasite shares antigens with another protozoan.

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